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نموذج رقم (18)
اقرار والتزام بالمعايير الأخلاقية والأمانة العلمية
وقوانين الجامعة الأردنية وأنظمتها وتعليماتها
لطلبة الماجستير

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عنوان الرسالة: The effect of flavonoid (Naringenin) on oxidative stress and inflammation: Role in atherosclerosis

اعلن بأنني قد التزمت بقوانين الجامعة الأردنية وأنظمتها وتعليماتها وقراراتها السارية المفعول المتعلقة باعداد رسائل الماجستير عندما قمت شخصيا" باعداد رسالتي وذلك بما ينسجم مع الأمانة العلمية وكافة المعايير الأخلاقية المتعارف عليها في كتابة الرسائل العلمية. كما أنني أعلن بأن رسالتي هذه غير منقولة أو مستلة من رسائل أو كتب أو أبحاث أو أي منشورات علمية تم نشرها أو تخزينها في أي وسيلة اعلامية، وتأسيسا" على ما تقدم فانني أتحمل المسؤولية بأنواعها كافة فيما لو تبين غير ذلك بما فيه حق مجلس العمداء في الجامعة الأردنية بالغاء قرار منحي الدرجة العلمية التي حصلت عليها وسحب شهادة التخرج مني بعد صدورها دون أن يكون لي أي حق في التظلم أو الاعتراض أو الطعن بأي صورة كانت في القرار الصادر عن مجلس العمداء بهذا الصدد.

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THE EFFECT OF FLAVONOID (NARINGENIN) ON OXIDATIVE STRESS AND INFLAMMATION: ROLE IN ATHEROSCLEROSIS

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COMMITTEE DECISION

This thesis / Dissertation **(The effect of Flavonoid (Naringenin) on oxidative stress and inflammation: Role in atherosclerosis)** was successfully Defended and Approved on July, 22, 2012.

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DEDICATION

This thesis is dedicated to my parents, my beloved husband Safwan and my little princess Leanna.

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List of abbreviations

CCR-2	Chemokine (C-C motif) receptor 2
CD14	Cluster of differentiation 14
CD68	Cluster of differentiation 68
CHD	Coronary heart disease
COX	Cyclooxygenase
CVD	Cardiovascular disease
EBM-2	Endothelial cell basal media-2
EGM-2	Endothelial cell growth media-2
HAEC	Human aortic endothelial cells
HO-1	Hemeoxygenase 1
ICAM-1	Intercellular adhesion molecule-1
IKKα	Inhibitor of nuclear factor kappa-B kinase subunit beta
IKKβ	Inhibitor of nuclear factor kappa-B kinase subunit beta
IL-1	Interleukin-1 beta
IL-6	Interleukin-6
IRS-1	Insulin receptor substrate 1
IκBα alpha	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor,
JNK-1	Mitogen-activated protein kinase 8
LBP	LPS binding protein
LDL	Low-density lipoprotein
LOX	Lipoxygenase

LPS	Lipopolysaccharides
MCP-1	Monocyte chemotactic protein-1
MNC	Peripheral blood mononuclear cell
MOP	Myeloperoxidase
NADPH	Nicotinamide-adenine dinucleotide phosphate
NFkB	Nuclear factor k B
NO	Nitric oxide
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase-Polymerase chain reaction
SOCS-3	Suppressor of cytokine signaling 3
SOD	Superoxide dismutases
THP-1	Human acute monocytic leukemia cell line
TLR-4	Toll-like receptor 4
TNF-α	Tumor necrosis factor- α
TXN2	Thioredoxin-2
VCAM-1	Vascular cell adhesion molecule-1
VLDL	Very-low-density lipoprotein

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ABSTRACT

Background: Oxidative stress and inflammation have been associated with several diseases such as type 2 diabetes, insulin resistance and atherosclerosis. It has been shown that macronutrients induce oxidative stress and inflammation. The aim of this study is to determine the effect of naringenin, a citrus flavonoid found in grapefruits and oranges, on oxidative stress and inflammation induced by macronutrients (glucose or fat) in cells involved in atherogenesis such as monocytes and endothlial cells.

Research design and methods: THP-1 cells and HAEC cells were challenged with 15 mM of glucose or cream in the presence or absence of 100μM of naringenin. The mRNA expression of several genes such as IL-1β, $p47^{phox}$, HO-1, MCP-1, CCR-2, TLR-4 and VCAM-1 were measured using Real-Time PCR. Also, protein expression of $p47^{phox}$ in THP-1 cells was measured using western blotting.

Results: Naringenin suppressed glucose and cream induced IL-1β, CCR-2 and MCP-1expression in THP-1 cells. In addition, the TLR-4 receptor was also suppressed in HAEC cells. On the other hand, naringenin increased HO-1 expression (antioxidant enzyme) significantly in THP-1 and HAEC cells. There was no change in the protein expression of the NADPH oxidase subunit $p47^{phox}$ in THP-1 cells. However, naringenin reduced the expression of VCAM-1 in HAEC when challenged with glucose but not cream.

Conclusion: Naringenin has neutralizing effects on macronutrient-induced oxidative stress and inflammation and may have a potential antiatherogenic effect.

INTRODUCTION

Flavonoids are a large group of polyphenolic compounds found in many plant-based foods regularly consumed by humans such as fruit, vegetables, grains, bark, roots, stems, flowers, tea, and wine. There are more than 4,000 different species of flavonoids in our diet that are divided into several subclasses based on their molecular structure. These naturally occurring compounds have shown a wide range of biological activities that have been attributed to their antioxidant (Dusan and Vesnak, 2007) and anti-inflammatory properties (Lotito and Frei, 2006). Among the various types of flavonoids, naringenin, found especially in many citrus fruits, has been shown to possess antioxidant (Cavia-Siaz *et al*., 2010), anti-inflammatory (Freischmidt *et al*., 2011) and antimutagenic properties (Ekambaram *et al*., 2008). It is derived from the deglycosylation of naringin, the main cause of bitterness in some citrus juices, by the action of enzymes such as α-rhamnosidase and β-glucosidase. Several studies detected naringenin and its metabolites but not naringin in human urine and plasma after oral administration of naringin (Gardana *et al*., 2007, Mennen *et al*., 2008).

Oxidative stress and inflammation have been linked with several human diseases such as cancer (Lau *et al*., 2008), type 2 diabetes (Muhammad *et al*., 2009), atherosclerosis (Hulsmans and Holvo *et al*, 2010) and neurological disorders (Bolanos *et al*., 2009). Oxidative stress results from the imbalance between generation of reactive oxygen species (ROS) and their degradation by antioxidants while inflammation is caused by microbial and viral infections, exposures to allergens, radiation, toxic chemicals, autoimmune and chronic diseases, obesity, alcohol, tobacco use and high caloric diet. It

has been suggested that pro-oxidative pro-inflammatory state in obesity and Type 2 diabetes may be related to chronically increased macronutrient intake (Ghanim *et al.*, 2004). Indeed macronutrients intake has been shown to induce oxidative stress and inflammation and may contribute to atherosclerosis and insulin resistance (Dandona *et al.*, 2005). For example, Deopurkar *et al.*, (2010) has shown that the intake of 300 kcal of glucose or saturated fat as cream induced significant increase in inflammatory response characterized by an increase in NF-κB binding in monocytes and the expression of two cytokines IL-1β and TNF-α. Also, glucose intake as well as lipid and protein cause an increase in ROS generation by leukocytes (Mohanty *et al.*, 2002). As for the intake of real food such as mixed meal (composed of carbohydrates, lipids and proteins) or high-fat high-carbohydrate (HFHC) meal they caused an increase in ROS generation, $p47^{phox}$ subunit expression as well as DNA-binding activity of NF-κB in monocytes (Aljada *et al.*, 2004).

It has been previously shown that drinking orange juice with HFHC neutralizes the pro-inflammatory effect of the meal and causes no change in $p47^{phox}$ concentration in monocytes (Ghanim *et al.*, 2010). In addition, the intake of just orange juice resulted in no change in ROS generation and NF-κB binding (Deopurkar *et al.*, 2010). This protective effect of orange juice is probably attributable to its flavonoids, naringenin and hesperetin, since they exert ROS suppressive and anti-inflammatory effects.

On the basis of the above, we were interested in investigating the effect of naringenin on macronutrient induced oxidative stress and inflammation in cells that are involved in atherogenesis such as monocytes and endothelial cells.

Therefore, we studied the expression level of :

1. IL-1β in Human acute monocytic leukemia cell line (THP-1) as a marker for inflammation.
2. $p47^{phox}$ a key protein of NADPH oxidase, the enzyme responsible for the formation of superoxide radical from molecular O_2, as an oxidative stress marker in THP-1 cells.
3. Both chemokine receptor CCR-2 in THP-1 as well as its ligand monocyte chemotactic protein-1 (MCP-1) in endothelial cell line (HAEC) since they play important roles in recruiting monocytes to subendothelial space which is central to atherosclerosis development.
4. Vascular cell adhesion molecule-1 (VCAM-1) in HAEC to which monocytes attach in the initial stage of atherosclerosis.
5. Hemoxygenase-1 (HO-1) as an antioxidant enzyme.
6. Toll like receptor 4 (TLR-4) in HAEC since it is involved in the pathogenesis of atherosclerosis and is expressed in atherosclerotic plaques.

On the basis of the above, we hypothesize that narigenin reduces basal and macronutrient (glucose, cream) induced oxidative stress and inflammation in THP-1 cells and HAEC and increases the expression of antioxidant enzyme (HO-1).

LITERATURE REVIEW

1. Naringenin

1.1 Flavonoids overview

Flavonoids are awidely distributed naturally occurring compounds found in fruits, vegetables, herbs, and beverages, such as tea and wines as flavonoid glycosides (Felgines *et al*., 2000, Wilcox *et al*., 1999). They are a subclass of polyphenols, which are characterized as containing two aromatic rings bound together by three carbon atoms that form an oxygenated heterocycle (ring C) (Manach *et al*.,2004). Flavonoids are further divided into subclasses based on the connection of the B ring to the C ring, the oxidation state and functional groups of the C ring (Beecher, 2003). The subclasses are flavonols, flavones, flavanones, catechins, anthocyanidins, isoflavones, dihydroflavonols, and chalcones. There are more than 4000 different flavonoids in the human diet, where the daily intake ranges from 1 mg to 23 mg (Wilcox *et al*., 1999).

Several epidemiological studies have proposed an inverse relationship between flavonoid intake and coronary heart disease (CHD) (Keli *et al*., 1996, Rimm *et al*., 1996). Therefore, there is an increasing interest in studying the possible antiatherogenic activity of flavonoids.

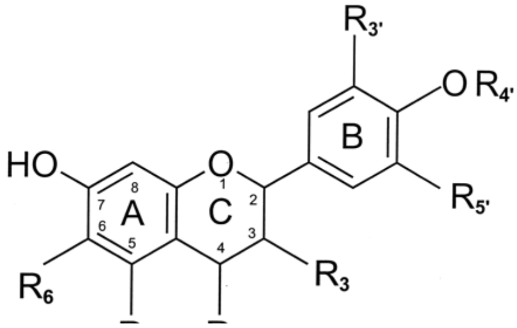


Figure 1: General structure and numbering pattern for common food flavonoids(Beecher, 2003)

Table 1: Flavonoid subclasses, their chemical characteristics, names of prominent food flavonoids and typical food sources (Beecher, 2003)

Flavonoid subclass	B-ring connection to C ring (position on C ring)	C-ring unsaturation	C-ring functional groups	Prominent food flavonoids[2]	Typical rich food sources
Flavanol	2	Non	3-Hydroxy 3-O-gallate	(+)-Catechin (C) (+)-Gallocatechin (GC) (+)-Gallocatechin (GC) (-)-Epicatechin-3-gallate (ECG) (-)-Epigallocatechin-3-gallate (EGCG)	Teas, red grapes and red wines
Flavanones	2	Non	4-Oxo	Eriodictyol Hesperetin Naringenin	Citrus fruits and juices, e.g., oranges, grapefruits, lemons
Flavones	2	2-3 Double bond	4-Oxo	Apigenin	Green leafy spices, e.g., Parsley
Isoflavones	3	2-3 Double bond	4-Oxo	DaidzeinGenistein Glycitein	Soybeans, soy foods and legumes

				Biochanin A Formononentin	
Flavonols	2	2-3 Double bond	3-Hydroxy 4-Oxo	Isorhamnetin Kaempferol Myricetin Quercetin	Nearly ubiquitous in foods, e.g., Quercetin
Anthocyanidins	2	1-2, 3-4 Double bonds	3-Hydroxy	Cyanidin Delphinidin Malvidin Pelargonidin Petunidin Peonidin	Red, purple and blue berries

1.2 Naringenin Chemistry

Naringenin (*2,3-dihydro-5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one)*, a citrus flavonoid that belongs to the flavanones class, has received considerable attention regarding its possible role in preventing and treating diseases such as cancer (Ekambaram *et al*., 2008) and atherosclerosis(Lee *et al*., 2001). There are several studies investigating the potential antioxidant and anti-inflammatory activity of this compound (Cavia-Siaz *et al*., 2010, Freischmidt *et al*., 2011). It is found mainly in citrus fruits such as grapefruits and oranges at a concentration of 53mg/100g and 11.15mg/100g of edible portion, respectively (U.S Department of Agriculture data base, 2003). It has a molecular weight of 272.26 (C15H12O5). Naringenin is derived from the deglycosylation of glycone naringin or narirutin. It is almost water insoluble and soluble in the organic solvents (O'Neil *et al*., 2006). On the other hand naringin (*naringenin-7-rhamnoglucoside)* is relatively water soluble and gives the bitter taste for grapefruit (O'Neil *et al*., 2006).

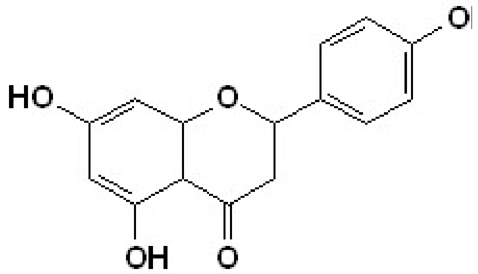


Figure 2: Structure of Naringenin (Vila-Real et al.,2011)

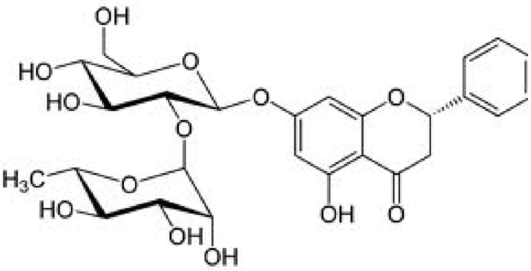


Figure 3: Structure of Naringin (Vila-Real et al.,2011)

1.3 Naringenin metabolism and bioavailability

All flavonoids except flavanols are found in nature bound to one or more glucose molecule. The glycosidic form of flavonoids cannot be absorbed by the small intestine (Manach *et al*., 2004). Naringin is the glycosidic form of naringenin that is bound to the B-glycoside (Wilcox *et al*., 1999). Naringin is not detected in either human or animal urine (Felgines *et al*., 2000, Furuta and Kasuya, 2000). On the other hand, several studies detected naringenin in human urine and plasma (Gardana *et al*., 2007, Mennen *et al*., 2008). These results show that naringin can be absorbed by the small intestine only if it is hydrolyzed to the aglycone form.

It has been shown that naringin is deglycosylated by intestinal enzymes or colonic microflora before they can be absorbed. The glycoside naringin that hasn't been absorbed by the small intestine will reach the colon and the microflora will hydrolize it to aglycone form (Manach *et al*., 2004). Several studies have shown the ability of intestinal microflora to cleave glycosidic bonds of naringin and release the aglycone form naringenin (Fuhr and Kummert, 1995). Therefore, the presence or absence of certain bacterial strains in our intestine will probably cause the interindividual variations in

transforming naringin into naringenin (Erlund *et al*., 2001). Intestinal microflora can both hydrolyze naringin and extensively metabolize the aglycone naringenin into various aromatic acids (Manach *et al*., 2004). This has been demonstrated in several studies on rats and human. For example, in human naringin can be metabolized by intestinal bacteria to naringenin and then to 4-hydroxybenzoicacid, phloroglucinol, 2,4,6-trihydroxybenzoic acid, and 4-hydroxyphenylacetic acid (Hodgson *et al*, 1998). On the other hand, naringin in rats is metabolized to 4-hydroxyphenylpropionicacid, 4-hydroxycinnamic acid, and 4-hydroxybenzoic acid sulfate (Wang *et al*., 2006) which indicates that different species differ in their intestinal micrflora metabolism of naringin and naringenin.

Once flavonoids are absorbed, they will undergo conjugations in the intestine and later in the liver. There are three kinds of conjugation: methylation, glucuronidation, and sulfation. As for naringenin, it is subject to glucuronidation, and sulfation but not methylation because naringenin contains no catechol groups and therefore it is not a substrate for catechol-O-methyltrasferase (Felgines *et al*., 2000). It has been shown that UDP-glucuronosyltrasferase catalayze the transfer of glucuronic acid from UDP-glucuronic acid to naringenin and thus forming naringeninglucuronoid (Green *et al*., 1998). This compound has been reported as well as naringenin sulfate in urine and plasma. Wang *et al*., (2006) has shown that naringenin sulfate is the major metabolites in the blood stream whether naringenin was given orally or intravenously to the rats. These metabolites circulate in our body bound to albumin and are excreted via two pathways ie, the biliary and the urinary route.

2. Oxidative stress and inflammation

2.1 oxidative stress

Oxidative stress and inflammation have gained a considerable attention regarding their association with several chronic diseases. It has been shown in several studies that oxidative stress and inflammation are linked to many human diseases such as cancer (Lau *et al*., 2008), type 2 diabetes (Muhammad *et al*., 2009, Dandona *et al*., 1996), atherosclerosis (Hulsmans and Holvo *et al*., 2010) and neurological disorders (Bolanos *et al*., 2009).

Oxidative stress can be defined as an imbalance between production of reactive oxygen species (ROS) and their degradation by antioxidants (Simone *et al*., 2010). This term was first coined by Helmut Sies in 1986 referring to the imbalance that arises when exposure to oxidants changes the normal redox status of major cellular antioxidants especially glutathione (Surh and Packer, 2005).

Oxidants (free radicals) such as superoxide anion, hydroxyl radical, hydrogen peroxide and organic peroxides are atoms or groups of atoms with one or more unpaired electrons. These atoms are unstable and very reactive in order to gain an electron from other compounds to become stable (Surh and Packer, 2005). These oxidants can be formed either normally in our body as a result of mitochondrial oxidative metabolism (Nemoto *et al*., 2000) and immunological defense (Victor *et al*., 2004) or due to environmental factors such as pollution, radiation, cigarette smoking and herbicides (Catin, 2010). Once free radicals are formed the antioxidants will try to eliminate them by one or more of the following mechanisms: metal chelation, scavenging free radicals, acting as chain

breaking and regulating gene expression (Surh and Packer, 2005). Antioxidants can be either compounds from our food such as polyphenols, flavonoids, carotenoids, vitamin C and E or enzymes in our body such as catalase, superoxide dismutase, glutathione and thioredoxin peroxidasis. If our body couldn't neutralize the oxidants, oxidative damage will affect important biomolecules such as lipids (Yager *et al*., 2010), proteins (Norris and Giasson, 2005) and DNA (Cooke *et al*., 2003). This oxidative damage has a key role in the pathogenesis of several diseases.

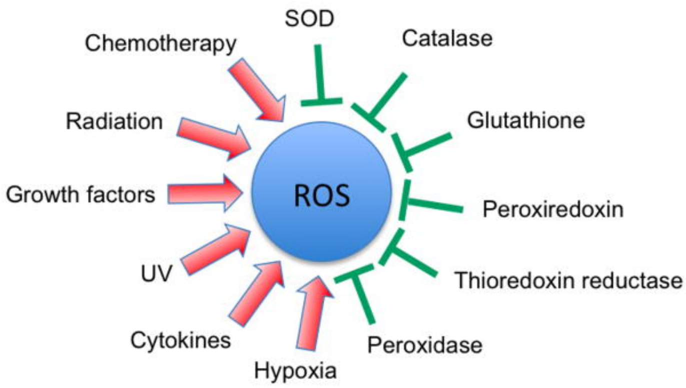


Figure 4: Schematic representation of various activators and inhibitors of reactive oxygen species production (Simone *et al*., 2010)

2.2 Inflammation

Inflammation is a protective tissue response to injury or destruction of tissues, which serves to destroy, dilute, or wall off both the injurious agent and the injured tissues. The classical signs of acute inflammation are pain, heat, redness, swelling, and loss of function. Celsius in the early first century was the first to describe the four signature signs of inflammation: redness, swelling, heat and pain. The fifth sign was described by Galen

and many authors over the centuries. In the nineteenth century, Lobstien, Bernard and Vichow described the macroscopic and microscopic features of inflammation (Wick and Grundtman, 2011).

The sources of inflammation are wide spread and include microbial and viral infections exposures to allergen, radiation, toxic chemicals, autoimmune and chronic diseases, obesity, alcohol, tobacco use, high caloric diet (Aggarwal *et al*., 2009, Schetter *et al*., 2010). Inflammation has two stages: acute and chronic inflammation. Acute inflammation which last for a short period of time is the initial stage of inflammation, mediated through the immune system and cause no harm to the host. On the other hand, when inflammation persists for a long period the host will be predisposed to chronic illness such as diabetes, cancer and atherosclerosis. This stage is called chronic inflammation. During inflammation mast cells and leukocytes are recruited to the site of damage which leads to a 'respiratory burst', increased release and accumulation of ROS at the site of damage (Coussens and Werb, 2002, Hussian *et al*., 2003). Inflammatory cells also produce soluble mediators, such as metabolites of arachidonic acid, cytokines (IL-1,6 and TNF-α) and chemokines (MCP-1), which act by further recruiting inflammatory cells to the site of damage and producing more reactive species (Simone *et al*., 2010). These key mediators can activate signal transduction cascades as well as induce changes in transcription factors, such as nuclear factor kappa B (NF-κB) and NF-E2 related factor-2 (Nrf2), which mediate immediate cellular stress responses (Simone *et al*., 2010).

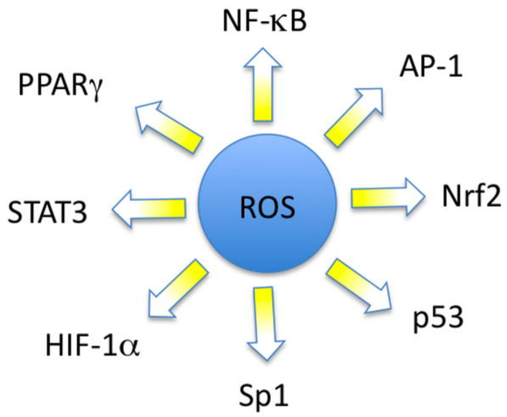


Figure 5: Schematic representation of various transcription factors that are modulated by reactive oxygen species (Simone *et al.*, 2010)

2.3 Macronutrients induce oxidative stress and inflammation

Macronutrients intake has been shown to induce oxidative stress and inflammation and may contribute to atherosclerosis and insulin resistance. For example, Deopurkar *et al.* (2010) has shown that the intake of 300 kcal of glucose or saturated fat as cream induced significant increase in inflammatory response characterized by an increase in NF-kB binding in monocytes and the expression of two cytokines IL-1B and TNF-α. Also, glucose intake as well as lipid and protein cause an increase in ROS generation by leukocytes (Mohanty *et al.*, 2002). As for the intake of real food such as mixed meal (composed of carbohydrates, lipids and proteins) or high-fat high-carbohydrate (HFHC) meal they caused an increase in ROS generation, $p47^{phox}$ subunit expression as well as DNA-binding activity of NF-kB in monocytes (Aljada *et al.*, 2004, Patel *et al.,* 2007). On the other hand, drinking orange juice with HFHC neutralized the proinflammatory effect of the meal and caused no change in $p47^{phox}$ concentration in monocytes (Ghanim *et al.*, 2010). Furthermore, it has been shown that the intake of just orange juice resulted

in no change in ROS generation and NF-kB binding (Deopurkar *et al*., 2010). On the basis of the above, it is of clinical importance to further study the mechanisms underlying postprandial oxidative stress and inflammation.

2.4 Oxidative stress and inflammation and their association with diseases

2.4.1 Oxidative stress and inflammation and type 2 diabetes:

Diabetes is the most frequently encountered metabolic disorder and its prevalence is increasing due to population growth, aging, urbanization, increasing prevalence of obesity and physical inactivity (Wild *et al*., 2004). The prevalence of diabetes for all aged group worldwide was 2.8% in 2000 and will be 4.4% by 2030 (Wild *et al*., 2004). Type 2 diabetes involves abnormalities at the receptor level resulting in insulin resistance and inadequate insulin secretions due to partial β cell failure (Saltiel and Kahn, 2001).

Several epidemiological studies have documented the association of obesity with type 2 diabetes and cardiovascular diseases (Kurth *et al*., 1983, Calle *et al*., 2002). Obesity and type 2 diabetes have been associated with inflammation and oxidative stress (Dandona *et al*., 1998, Higdon *et al*., 2003). Insulin resistance is considered to be a major risk factor for type 2 diabetes. It is characterized by less than optimal response to insulin through the insulin receptor signaling pathway. It has been shown that oxidative and inflammatory pathways contribute significantly to the interruption of insulin signaling thus inducing insulin resistance. For example, pro-inflammatory kinases such as JNK-1(Lee *et al*., 2003), IKKβ (Arkan *et al*.,2005) and SOCS-3 (Ghanim *et al*.,2009) are known to interfere with IRS-1 phosphorylation by insulin leading to suppressed insulin effect. It

has been suggested that pro-oxidative and pro-inflammatory state in obesity and type 2 diabetes may be related to chronically increased macronutrient intake (Ghanim *et al*., 2004). Indeed macronutrientsintake has been shown to induce oxidative stress and inflammation and may contribute to insulin resistance (Dandona *et al*., 2005).

2.4.2 Oxidative stress and inflammation and atherosclerosis

Atherosclerosis is a chronic inflammatory state of the vessel wall (Ross, 1999). During atherogenesis, the activated monocyte adheres to the endothelium following endothelial damage through specific adhesion molecules on the endothelium and their respective ligands on the leukocytes. It then moves into the sub-endothelium where it is believed to change its phenotype into the macrophage and to take up oxidized LDL. An excess of this lipid load causes a change in its appearance into a foam cell. Clusters of foam cells form the initial lesion of atherosclerosis, the fatty streak (Ross, 1999). Understanding the pathophysiology of atherosclerosis occurred around the turn of the millennium. During most of the twentieth century, atherosclerosis considered a degenerative disease, mostly related to aging. In 1999, Russell Ross discovered that atherosclerosis is an inflammatory disease and made a big shift in understanding this disease. (Wick and Grundtman, 2011)

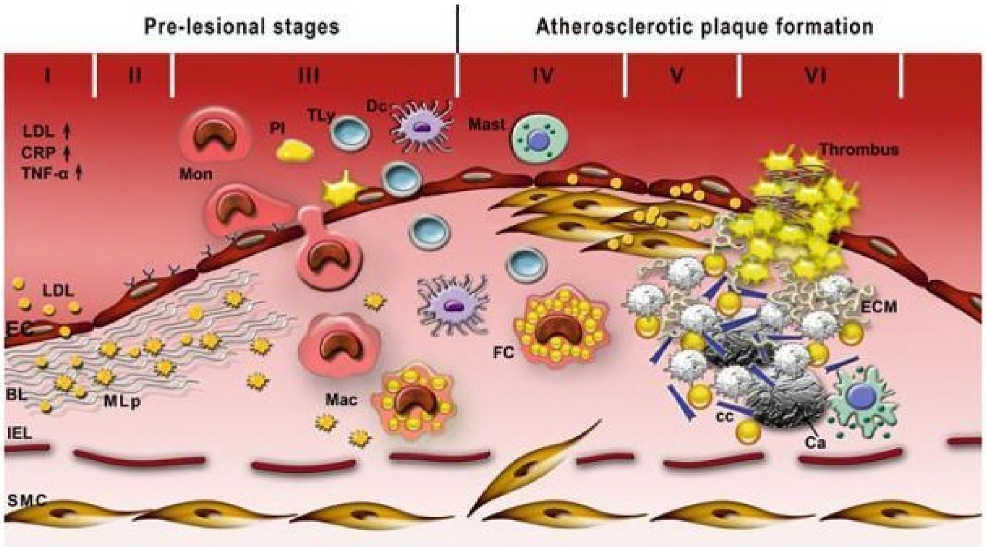


Figure 6: Diagramatic representation of the atherosclerotic plaque formation(Wick and Grundtman, 2011)

Recently, chronic diseases are the major contributors to total global mortality. The total number of cardiovascular disease (CVD) deaths had increased globally from 14.4 million in 1990 to 17.5 million in 2005. More than 80 percent of the deaths occurred in low and middle income countries. The World Health Organization estimates there will be about 20 million CVD deaths in 2015, accounting for 30 percent of all deaths worldwide. Thus, CVD is today the largest single contributor to global mortality and will continue to dominate mortality trends in the future. (Valentín and Bridget, 2010)

Atherosclerosis is the underlying cause for most CVD (Wick and Grundtman, 2011). Obesity and type 2 diabetes are associated with increased risk of atherosclerosis. Also, macronutrients intake has been shown to induce oxidative stress and inflammation and may contribute to atherosclerosis. For example, it has been shown that the intake of a high fat high carbohydrate (HFHC) meal in human results in an increase in ROS generation, $p47^{phox}$ expression, intranuclear NFκB binding, IKKα and β expression and activity and a decrease in IκBα expression in MNC (Dandona *et al.*, 2005). This is

accompanied by an increase in TNFα and IL-1β expression in MNC (Dandona *et al*., 2005). Furthermore, this response is associated with an increase in plasma concentrations of endotoxin (LPS) and LPS binding protein (LBP) and the expression of TLR-4 and CD14 (Ghanim *et al*., 2009). Thus, postprandial oxidative and inflammatory stress is associated with endotoxemia and the expression of its receptor, TLR-4 (Ghanim *et al*., 2009).

Furthermore, postprandial lipemia has been correlated with increased risk of atherosclerosis and cardiovascular disease (Hyson *et al*., 2003). In addition to the effect of lipids on the vasculature and platelets activity, lipid intake induces activation of leukocytes which triggers endothelium adhesion. It has been shown that a single challenge with 33g of dairy cream induces an increase in ROS generation (Mohanty *et al*., 2002) and inflammation in the MNC (Deopurkar *et al*., 2010). It was demonstrated that the intake of cream results in an increase in intracellular lipids in the MNC as well as an increase in monocyte to macrophage differentiation markers including CD68 (unpublished data, submitted). These data show for the first time that fat intake results not only in an increase in inflammation, oxidative stress and plasma lipid concentrations but also an increase in the intracellular lipid concentrations. The increase in lipid in MNC is of great interest since several cells contained in this fraction are involved in atherogenesis, in particular, the monocytes.

3. Naringenin roles in atherosclerosis

3.1 Naringenin, antioxidant role in atherosclerosis

Flavonoids intake have been associated with decreased risk of cardiovascular diseases (Arts and Hollman, 2005) attributed to their antioxidant property (Saija*et al*., 1995). It has been shown that naringenin and hesperitin reduce reactive oxygen species (ROS) by 77±8% and 52±7%, respectively in mononuclear cells (MNC) *in vitro* (Ghanim *et al*., 2007). ROS can be generated by cells of the vascular wall such as macrophages, endothelial cells and smooth muscle cells (Loscalzo, 2005). These cells can oxidize or modify low density lipoprotein which is the early event in the formation of foam cells and pathogenesis of atherosclerosis (Willox *et al*., 1999). Flavonoids have four possible antioxidant mechanisms in order to prevent or reduce the formation of reactive species: 1) chelating transition metals, 2) direct scavenging of free radicals, 3) inducing phase 2 enzymes and 4) targeting receptors and enzymes involved in cell signaling, transduction and gene expression (Surh and Packer, 2005).

It has been shown that flavonoids have the ability to chelate metals (Mira *et al*., 2002, Dusan and Vesnak, 2007). The transition metals such as iron and copper are an important catalyst for the oxidation reaction and their chelation will prevent the formation of free radicals.

Several studies suggested that naringenin have a direct scavenging activity of oxygen and nitrogen free radicals such as hydroxyl, superoxide and nitric oxide radicals. For example, Khanduja *et al*. (2003) has shown that naringenin is able to scavenge free radicals in vitro. Cavia-Siaz *et al*. (2010) has studied the scavenging activity of both

naringenin and narigin and found that naringenin exhibited higher antioxidant capacity and hydroxyl and superoxide radicals scavenger efficiency than naringin in rats.

As for the induction of phase 2 enzymes such as HO-1, SOD, TXN and catalase, Lee *et al*. (2002) has investigated the effect of high cholesterol diet on the antioxidant enzyme activities of erythrocytes and liver. He has shown that naringenin significantly increased the activity of catalases and SOD in erythrocytes and liver. Lin *et al*. (2005) demonstrated that naringenin increases HO-1 protein expression in the presence or absence of LPS and showed time and dose dependent inhibition of LPS-induced NO production in macrophages.

In addition to naringenin direct antioxidant roles, they may inhibit the oxidation of LDL by mechanisms involving inhibition of lipoxygenase (LOX), cyclooxygenase (COX) and myeloperoxidase (MPO).It has been suggested that lipoxygenase (LOX) is involved in the early events of atherosclerosis by inducing plasma LDL oxidation in the subendothelial space of the arterial wall (Da Silva *et al*., 1998). Corvazier and Maclouf (1985) demonstrated that naringenin (500 mM) is an irreversible inhibitor of both LOX and cyclooxygenase (COX) pathways. Furthermore, naringenin has been shown to inhibit myeloperoxidase (MPO) (Divi and Doerge, 1996). MPO secreted by macrophages and activated neutrophils is a potent catalyst of LDL oxidation in vitro and co-localizes with macrophages in human atherosclerotic lesions. MPO generates a range of reactive species, including the potent oxidizing agent hypochlorous acid. (Heinecke, 1997)

Several studies demonstrated the ability of naringenin in reducing lipid peroxidation. For example, naringenin significantly lowered circulatory lipid peroxide products in ethanol-

fed rats (Jayaraman and Namasivayam, 2011). Also, Lee *et al*. (2005) has shown the ability of naringenin to lower the plama lipids peroxide products in high cholesterol fed rats as compared to the control group.

3.2 Naringenin: anti-inflammatory role in atherosclerosis

Flavonoids have also been shown to have anti-inflammatory activities (Lotito and Frei, 2006). Since atherosclerosis is a chronic inflammation of the vessel wall, it is important to study the anti-inflammatory roles of naringenin with respect to atherosclerosis.

It has been shown that the initial step in atherogenesis is arterial leukocyte recruitment. Leukocyte – endothelial interaction and leukocyte emigration to the sub-endothelium are mediated by adhesion molecules expression on the endothelial cells, such as E- and P-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) (Blankenberg *et al*, 2003). It has been suggested that VCAM-1 and monocyte chemotactic protein-1 (MCP-1) play a very important role in the recruitment of monocytes in atherosclerosis. Lee *et al*., (2001) showed that naringenin and naringin decrease the expression of MCP-1 and ICAM-1 of the thoracic aorta in cholesterol fed rabbits compared to the control group. Also, it has been demonstrated that naringenin exerts moderate activity on TNF-α induced ICAM-1 expression in human microvascular endothelial cells (Freischmidt *et al*., 2011). Therefore, it is possible that atherosclerosis can be improved by down regulation of proatherogenic molecules such as MCP-1, VCAM-1 and ICAM-1.

Pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6 are thought to be involved in the initiation and amplification of the inflammatory processes by recruiting more leukocyte to cite of inflammation. They contribute to the pathophysiology of many

inflammatory diseases such as rheumatoide arthritis and atherosclerosis (Bodet *et al*., 2008). It has been shown that naringenin is a potent inhibitor of the pro-inflammatory cytokines (IL-1β, TNF-α and IL-6) response induced by lipopolysaccharide in both macrophages and whole blood (Bodet *et al*., 2008). Naringenin anti-inflammatory roles need further investigation in animals and human regarding its possible effect as anti-atherosclerotic compound.

In view of the postprandial existence of oxidative and inflammatory stress and its likely contribution to atherogenesis, it is important to investigate whether there are ways to reduce postprandial oxidative and inflammatory stress. One approach would be to identify foods which are not likely to induce such changes. Another approach would be to identify natural or pharmaceutical products which would significantly reduce basal and postprandial oxidative and inflammatory stress. Recently it has been shown that the intake of orange juice with the HFHC meal neutralizes/prevents the pro-inflammatory effect of meal (Ghanim *et al*., 2010). This effect of orange juice is probably attributable to its flavonoids, naringenin and hesperidin, since they exert ROS suppressive and anti-inflammatory effects (Ghanim *et al*., 2007).

The potent effects of flavonoids have been demonstrated in experimental animal models in relation to endotoxin induced inflammation and in cells, *in vitro*. Further studies demonstrating the anti-inflammatory effects of these flavonoids in the human, *in viv*o, are required. In addition the effect of these flavonoids, especially naringenin and hesperetin, on atherogenesis is not known. Therefore, we are investigating the effect of naringenin on neutralizing macronutrients induced oxidative stress and inflammation in monocytes and endothelial cells since these cells are involved in atherogenesis.

On the basis of the above, we hypothesize that narigenin reduces basal and macronutrient (glucose, cream) induced oxidative stress and inflammation in THP-1 cells and HAEC.

METHODS AND MATERIALS

1. Experimental design:

In this study we utilized an in vitro model to investigate the effect of Naringenin (Safc Supply Solutions, USA) on cells involved in atherogenesis (i.e monocytes and endothelial cells) after challenging them with macronutrients such as a high concentration of glucose (Sigma, USA) or Whipping cream (Tops, USA). The content of the dairy cream used includes 70% saturated fat, 28% unsaturated fat, <2% protein, and no carbohydrate. In our experiments we utilized a monocytic cell line, THP-1(Sigma, USA) at passage 12-14 and an endothelial cell line, HAEC (Lonza, USA) at passage 7. Each cell line was divided into six groups as the following: the first group is a control and received only 5mM of glucose to mimic *in vivo* normal glucose concentration and incubated for one hour. The second group received 100µM of naringenin plus 5mM of glucose and incubated for three hours. As for the third group they received a high concentration of glucose (15mM) and incubated for one hour since the glucose level reach the peek *in vivo* at 1 hour after glucose intake (Deopurkar *et al*., 2010). In the fourth group the cells were incubated for three hours with 20µl/ml of heavy whipping cream since triglycerides, Free fatty acids and VLDL reach the peak *in vivo* after three hours form cream intake (Deopurkar *et al*., 2010). Whereas the fifth group were incubated with 100µM of naringenin for three hours and then one hour with 15 mM of glucose. In the last group we incubated the cells for three hours with 100µM of naringenin and then for another three hours with 20µl/ml of heavy whipping cream.

2. Methods

2.1. HAEC culturing:

A frozen ampule of HAEC (0.5×10^6cells) were thawed in a water bath at 37°C and added to a 15 ml of Endothelial cell growth media (EGM-2)(Endothelial cell basal media (EBM-2), hydrocortisone, fetal bovine serum, human fibroblast growth factor-basic, vascular endothelial growth factor, insulin like growth factor , ascorbic acid, human epidermal growth factor, gentamicin, amphotericin and Heparin) (Lonza, USA) pre-warmed to 37°C in T75 culturing flask. The flask was incubated in a 5% CO_2 at 37°C till the cells reached 95% confluence (Examined under inverted microscope). The medium was changed the day after seeding and every other day and thereafter. The amount of medium that was added depended on cells confluence. After the cells reached 95% confluence they were trypsinized with 5ml of Trypsin-EDTA reagent (Lonza, USA) until 90% of cells became rounded as seen under the microscope. Then, they were neutralized with 10ml of Trypsin Neutralizing solution (Lonza, USA) and transferred to a 15ml centrifuge tube. The harvested cells were centrifuged at 220xg for 5 minutes to pellet the cells and the supernatant was aspirated. A 2ml of EGM-2 medium was added to the pellet, suspended and transferred to 6-wells culturing plate that contains 2ml of medium in each well. The cells were incubated till they reached 95% confluence and the experiments were performed as previously described after checking their viability using Trypan Blue solution (0.4%) (the average viability was 90%) (Sigma, USA).

2.2. THP-1 cells culturing:

A frozen ampule of THP-1 cells was thawed, added to 5ml of pre-warmed 20% RPMI medium (4.5 g/L D-glucose, 1.5 g/L Sodium Bicarbonate, 1 mM Sodium Pyruvate, 10 mM HEPES and 300 mg/L L-glutamine, fetal bovine serum, Phenol red) (Invitrogen, USA) in T25 culturing flask and incubated in a 5% CO2 at 37°C. The next day the cell suspension was centrifuged at 200xg in 15 ml tube for two minutes to pellet the cells and the supernatant was aspirated. Another 5ml of the medium was added to the pellet and incubated for seven days till they reached 12×10^6 cells. Each day the cells were checked and the culture maintained between $0.2\text{-}0.8\times10^6$ cells/ml by adding a 10% RPMI medium (Invitrogen, USA). Once the cells reached the desired number, they were split into 6 flasks and were grown to reach $8\text{-}10\times10^6$ cells in each flask. After that, cell's viability was checked using Trypan Blue (the average viability was 90%) and the experiments were done as previously described.

2.3. Preparation of total cell lysate:

The cells (3×10^6 THP-1 cells) were washed with one ml of Dulbecco's phosphate buffered saline (DPBS) (2.67mM Potassium Chloride, 1.47mM Potassium Phosphate monobasic, 139.9mM Sodium Chloride, 8.06mM Sodium Phosphate dibasic) (Invitrogen, USA) was added to wash the cells. Then the cells were frozen in liquid nitrogen and stored at -80°C. To the THP-1cell pellet , 50µl of lysis buffer (10mMTris-HCl, 150mMNaCl, 0.2% Triton-X and protease and phosphatases inhibitors) containing 1µl of 0.1M PMSF (87 mg of phenylmethylsulfonyl fluoride, 5 mL 2-propanol) (Sigma, USA) and 1µl of protease inhibitor cocktail was added (Sigma, USA) and incubated on ice for

30 minutes with occasional mixing. The lysate was centrifuged at 12000xg for 15 minutes at 4°C to pellet the non-soluble particles and cell debris and the supernatant was collected and stored at -80°C.

2.4. Total protein determination in cell lysate:

Protein concentration was determined using Bradford protein assay (Bradford, 1976). A 10μl of total cell lysate were pipetted into 40μl of water, mixed and a 20μl in duplicates were transferred to 2ml eppendorf tube. One ml of room temperature Bradford reagent (1X concentrated Coomassie Brilliant Blue G-250 dye) (Fermantas, USA) was added to each eppendorf tube. Measurement of the absorbance was taken within five minutes of Bradford addition at 595 nm against a blank of distilled water using Biospec-1601 spectrophotometer (Shimadzu corporation, MD).

2.5. Western blotting:

A solution of total cell lysate containing sixty μg of protein were boiled with 4X SDS buffer (100 mMTris-HCl, 4% SDS, 20% glycerol, 0.06% Bromophenol Blue and 200 mM DTT) at 3:1 ratio. The proteins were electrophoresed at 200 V for 35 minutes under denaturing conditions on ready to use 10% SDS-polyacrylamide gels (SDS-PAGE) (Bio-rad, USA) and then transferred to polyvinylidene difluoride (PVDF) membrane (Bio-rad, USA) by sandwiching the gel and membrane between two stacks of filter paper and placing them in direct contact with plate electrode of Trans-Blot SD machine (Bio-rad, USA) and run it for 20 minutes at 12 V (Bio-rad, USA) (Figure 7). Non-specific binding sites on the PVDF membranes were blocked by incubating them in 5% dry milk in 0.1%

Tween-TBS (TTBS) solution (10X TBS, 10% Tween) (Bio-rad, USA). Monoclonal mouse anti-human antibodies against $p47^{phox}$ (BD biosciences, USA) or polyclonal goat anti-human antibodies against actin (Santa cruz biotechnology, USA) were used one at a time in 5% dry milk in 0.1% TTBS and incubated for one hour. Membranes were washed 5 times for 5 minutes each with TTBS to remove the excess primary antibody. Then a species specific secondary antibody labeled with horseradish peroxidase (HRP) (Goat anti-mouse IgG-HRP or rabbit anti-goat IgG-HRP) (Santa cruz biotechnology, USA) was added to the membranes and incubated for one hour. After washing the membranes 5 times for 5 minutes each with TTBS we added 7.5ml of Super Signal West Pico Chemiluminescent substrate (Thermo scientific, USA) which contains luminol as asubstrate for HRP enzyme. This enzyme catalyzes the oxidation of luminol and produces a detectable light as a by-product. The light signal was captured on X-ray film (Kalamazoo X-ray Sales Inc, USA) (Figure 8). After that we scanned the film and we determined the density of the band using molecular analyst software (Bio-Rad, CA) and all the values were normalized to the actin quantity to ensure correction for the amount of total protein on the membranes in case of error or incomplete transfer (Figure 9, 10). After each labeling the membranes were stripped for 15 minutes at 37°C in Restore stripping buffer (Thermo scientific, USA) and used again in immunoblotting.

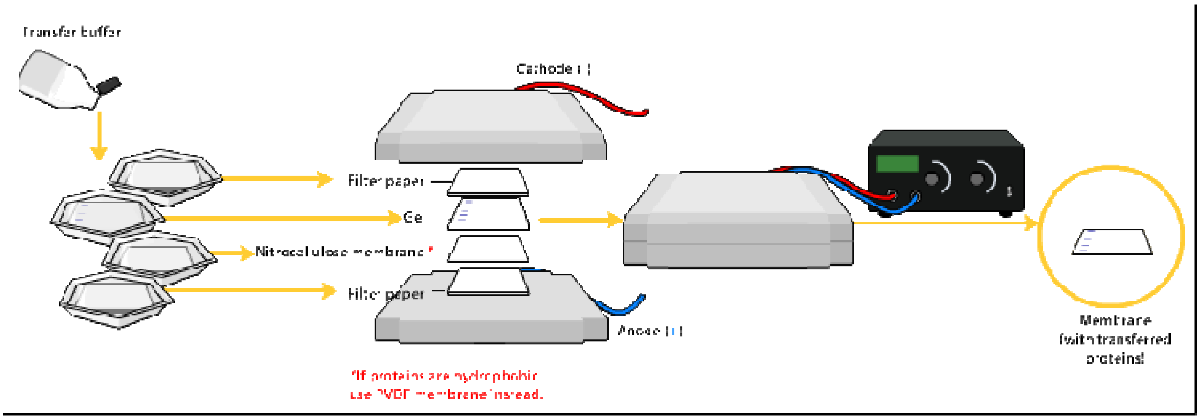


Figure 7: Transferring protein to polyvinylidene difluoride (PVDF) membrane.

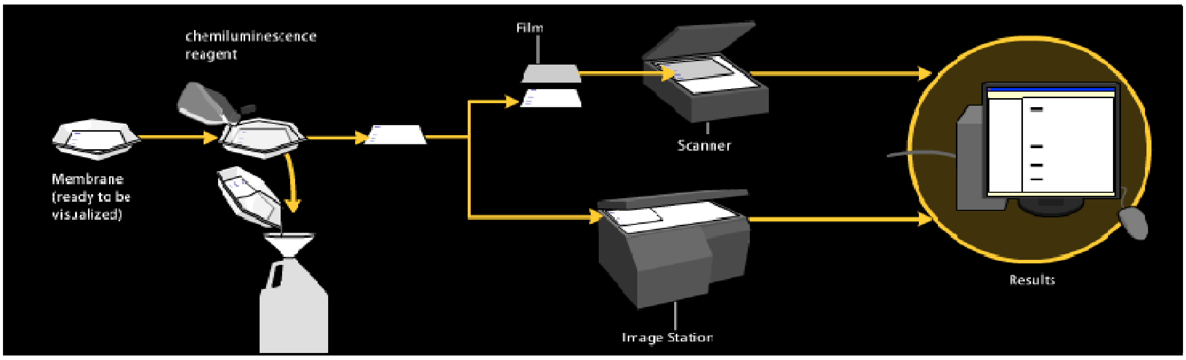


Figure 8: Chemiluminescent detection.

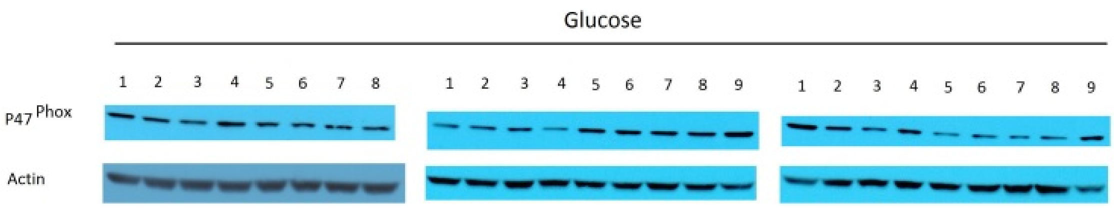


Figure 9: Protein expression of Actin and p47^{phox} in THP-1 cells determined by Western blotting (Glucose challenge). (1): Control, (3): Naringenin, (5, 8): Naringenin + 15mM glucose, (6, 9): 15mM glucose.

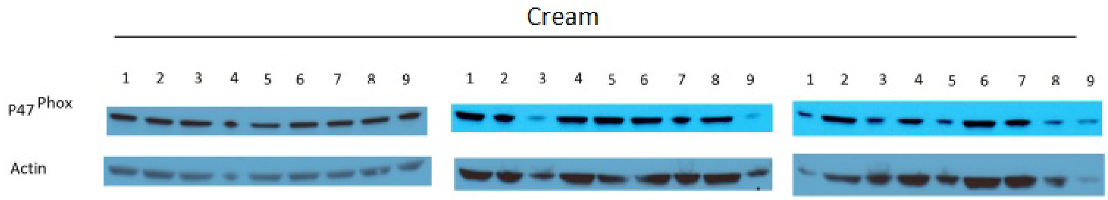


Figure 10: Protein expression of Actin and p47^{phox} in THP-1 cells determined by Western blotting (Cream challenge). (1): Control, (3): Naringenin, (4, 5): Cream, (8, 9): Naringenin + Cream.

2.6. Total RNA isolation:

One million cells of HAEC and 3*10^{6} THP-1cells were washed with one ml of Phosphate Buffered Saline. Cells were pelleted, then frozen in liquid nitrogen and stored at -80°C.Total RNA of both cell-types were isolated using RNAqueous-4PCR kit (Ambion, USA). Briefly, 500µl of Lysis/Binding solution was added to the thawed cells and vortexed vigorously for one minute. An equal volume of 64% Ethanol was added to the cells and mixed by inverting the tube. Then, lysate /Ethanol mixture was applied to a filter cartridge assembled in a collection tube (Ambion, USA) and centrifuged at 15,000xg for one minute. After that, washing was done 3 times using wash solutions and centrifugation was done after each wash at 15,000xg for one minute. Filter cartridge was transferred to a new collection tube and 50µl of preheated elution solution to 70°C was pipetted in the center of the filter and centrifuged at 15,000xg for 30 seconds to elute RNA.

2.7. RNA quantification and assessment of purity by UV absorbance:

The concentration and purity of RNA was determined by measuring it's absorbance at 260-280 nm using Biospec-101 spectrophotometer (Shimadzu corporation, MD) (Table 2, 3). Three µl of RNA was diluted in 57µl of 1XTE buffer (10mM Tris-HCl pH8, 1 mM EDTA) (Sigma, USA) and the absorbance was read against a blank of TE buffer. The ratio of A260 to A280 was used as a measure of RNA purity and it should fall in the range of 1.8-2.2.

Table 2: HAEC cells RNA concentration in µg/µl. Nar:Naringenin, HG: High concentration of glucose(15mM), Cr: Cream.

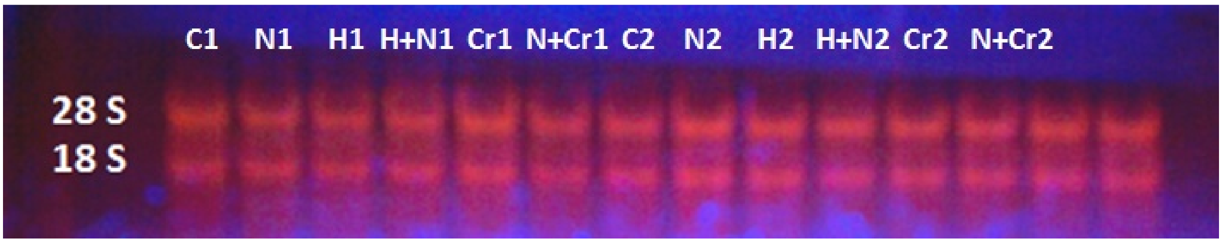
Sample	Concentration in µg/µl					
control	0.155	0.131	0.112	0.109	0.097	0.162
Nar	0.133	0.128	0.105	0.098	0.095	0.116
HG	0.089	0.110	0.136	0.106	0.117	0.134
Nar+HG	0.097	0.104	0.112	0.106	0.100	0.109
Cream	0.134	0.115	0.101	0.145	0.126	0.094
Nar+cr	0.135	0.114	0.071	0.109	0.110	0.108

Table 3: THP-1 cells RNA concentration in µg/µl. Nar:Naringenin, HG: High concentration of glucose(15mM), Cr: Cream.

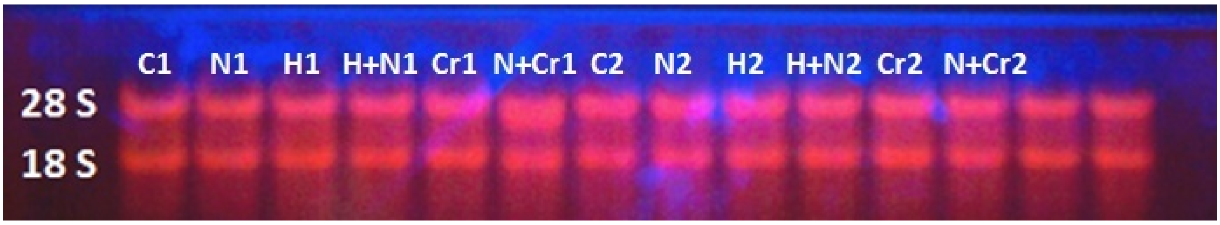
Sample	Concentration in µg/µl					
control	0.280	0.278	0.321	0.348	0.264	0.158
Nar	0.099	0.279	0.362	0.201	0.079	0.143
HG	0.264	0.047	0.087	0.249	0.032	0.255
Nar+HG	0.074	0.036	0.202	0.222	0.065	0.140
cream	0.275	0.314	0.347	0.165	0.315	0.320
Nar+cr	0.296	0.399	0.396	0.281	0.299	0.297

2.8. RNA electrophoresis:

The quality of the isolated RNA samples was assessed by electrophoresis on 1.2% denaturing agarose gel (Lonza, USA). A total volume of 5µl, which contains 0.2µg of RNA, 1.25µl of Formaldehyde Load dye (Lonza, USA) and water (To adjust the final volume to 5µl) was heated to 65°C for five minutes to denature the RNA and loaded to the gel. The gel was electrophoresed at 22V for 5 minutes and visualized on a UV transluminator. A good quality of RNA is determined by visualizing two sharp intense bands with motilities of about 5kb and 2kb corresponding to 28S and 18S ribosomal RNA, respectively (Figure 11).



(a)



(b)

Figure 11: Total RNA quality confirmation. (a) RNA (0.2µg) from THP-1cells and (b) RNA (0.02µg) from HAEC cells were separated on 1.2% denaturing agarose gel and viewed under UV light using Formaldehyde Load dye. C: control, N: naringenin, H: high Glucose, Cr: cream.

2.9. Reverse transcription:

Since RNA does not serve as a template for PCR, it must first be copied into cDNA by a reverse transcriptase. The generation of first strand cDNA from total RNA was performed by reverse transcription using the QuantiTect Reverse Transcription Kit (QIAGEN, USA). One µg of total RNA was mixed with 2µl of gDNA wipeout buffer in 14µl total volume and heated for two minutes at 42°C. The reaction mixture was continued at 42°C for 30 minutes in the presence of 4µl of 5X Quantiscript RT Buffer, 1µl of Quantiscript Reverse transcriptase and 1µl of RT primer mix (Oligo-dT and random primers). The reverse transcriptase was inactivated by heating for 3 minutes at 95°C and the reaction diluted to 100µl with RNase free water.

2.10. Real time PCR:

Gene expression level of IL-1β, MCP-1, $p47^{phox}$, HO-1, CCR-2, VCAM-1 and TLR-4 for the six groups listed before in the experimental design were determined by real time PCR technique and the QuantiTect SYBER Green PCR kit (QIAGEN, USA). The fluorescent dye SYBER Green in this kit binds to all double-stranded DNA emitting a fluorescent signal on binding. This enables the rapid analysis of many different targets without having to synthesize target specific labeled probes. Also, the use of the hot –start enzyme, HotStarTaq DNA polymerase provided by QuantiTect SYBER Green PCR kit (QIAGEN, USA), which has no activity at ambient temperature, allows for high specificity and prevents the formation of misprimed products and primers-dimers during reaction setup. Gene specific primer for CCR2 was obtained from published references (Vande Broek *et al.,* 2003) and for IL-1β, MCP-1, $p47^{phox}$, HO-1, VCAM-1 and TLR-4 were generated

using the 3' Primers software and then synthesized commercially (Life Technologies, USA). Real time PCR was done using (Mx3000P and Mx3005P QPCR systems Stratagene, USA) in which 2µl of cDNA, 10ul Sybergreen Master Mix, 0.5µl of gene specific primers and 7.5µl of RNase free water were mixed in specialized 200µl 8-strip PCR tubes with flat cap (Laboratory Products Sales Inc., USA) and placed in the cycler. The standard protocol that was used for all the genes consisted of three stages: stage one was an initial activation step for 15 minutes at 95°C; stage two, which consist of 40 cycles, has a two temperature cycling with denaturing at 95°C and annealing at the specific annealing temperature (Table 4) for each primer pair; and stage three was a melting (dissociation) curve analysis of the PCR product to verify specificity and identity. For each gene a standard curve was generated from a pooled sample prepared from the six groups listed in the experimental design and was used to obtain relative expression levels for all the samples. All values were normalized to (Ubiquitin C, Ribosomal Protein L13a, Beta-Actin and Isomerase A) expression levels, which were measured in duplicate to ensure accuracy.

Table 4 : List of Primers used in real time RT-PCR.

Gene	Primers sequence	Fragment length (bp)	Anneal-ing Temp C°
CCR-2 Forward	5' GATGGTCGTGGAAAAGGAAA 3'	203	60
CCR-2 Reverse	5' CAACCCAGCTGGAGTCTCTC 3'		
HO-1 Forward	5' CTTCTTCACCTTCCCCAACA 3'	165	57
HO-1 Reverse	5' AGCTCCTGCAACTCCTCAAA 3'		
IL-1β Forward	5' TACGAATCTCCGACCACCACTACAG 3'	295	60
IL-1β Reverse	5' TGGAGGTGGAGAGCTTTCAGTTCAATG 3'		
MCP-1 Forward	5' CCCCAGTCACCTGCTGTTAT 3'	171	62
MCP-1 Reverse	5' TGGAATCCTGAACCCACTTC 3'		
$p47^{pox}$ Forward	5' AGTCCTGACGAGACGGAAGA 3'	194	62
$p47^{pox}$ Reverse	5' TACATGGACGGGAAGTAGCC 3'		
TLR-4 Forward	5' TTGGGACAACCAGCCTAAAG 3'	210	60
TLR-4 Reverse	5' TGCCATTGAAAGCAACTCTG 3'		
VCAM-1 Forward	5' TCGAGATGAGTGGTGGCCTCGTGAAT 3'	170	59
VCAM-1 Reverse	5' GGAAAGCCCTGGCTCAAGCATGTCATAT 3'		

3. Statistical Analysis:

Statistical analysis was conducted with SigmaStat software version 3.1 (SPSS Inc, Chicago, IL). All data are expressed as mean ± S.E of arbitrary units and percent change is calculated from the means. Statistical Analysis was carried out using paired t-test between control and treated groups and Wilcoxon signed rank test when normality test failed. All the treated groups for each cell line were repeated 6 times (n=6).

RESULTS

1. IL-1β mRNA expression in THP-1 cells

Incubating THP-1 cells with naringenin at basal condition (5mM Glucose) caused a significant reduction in IL-1β expression by 41±7% (P=0.001). Challenging the cells with high concentration of glucose (15mM) caused significant increase by 86±17.6% (P<0.05) (Table 5). However, challenging the cells with 15mM of glucose in the presence of naringenin caused a significant reduction in IL-1β expression by 109± 17% (P=0.04) compared to IL-1β expression at high concentration of glucose (15mM). On the other hand, incubating THP-1 cells with cream alone caused a significant increase in IL-1β expression by 139±40% (P=0.042) which was suppressed by 45± 23% (P=0.015) when naringenin was added. (Figure 12)

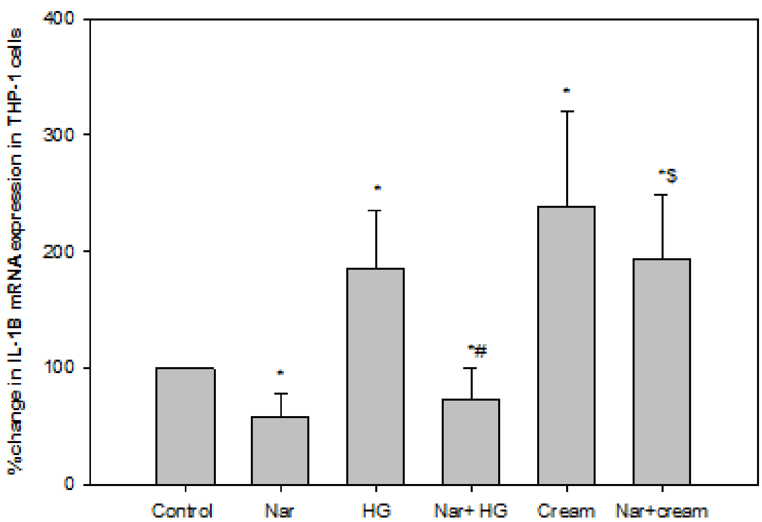


Figure 12: Percent change in mRNA expression of IL-1β in THP-1 measured by RT-PCR (n=6). Data are means ± SEM. *:P<0.05 compared to control; #:P<0.05 comparing HG with Nar+HG; $:P<0.05 comparing cream with Nar+cream , all the comparisons were made using paired t-test. Nar:Naringenin, HG: High concentration of glucose(15mM).

Table 5: Percent change in mRNA expression of IL-1β in THP-1 measured by RT-PCR (n=6).

Sample	**% change**	***P*-value**
Naringein	↓ 41%	.001
High concentration of glucose	↑ 86%	.002
Naringenin+ High concentration of glucose	↓ 26%	.040
Cream	↑ 139%	.040
Naringenin+cream	↑ 94%	.042

2. $p47^{phox}$mRNA expression in THP-1 cells

Challenging the THP-1 cells with high concentration of glucose (15mM) or cream resulted in a significant increase in $p47^{phox}$ expression by 71± 28% (P=0.04) and 54±11% (P=0.005), respectively (Table 6). On the other hand, naringenin showed a trend of reduction (P=0.18) in $p47^{phox}$ expression. Incubating THP-1 cells with 15mM of glucose or cream in the presence of naringenin caused no statistically significant change compared to control. However, adding naringenin to cream reduced the increase significantly by 51±14% (p=0.014) compared to the cream (Figure 13).

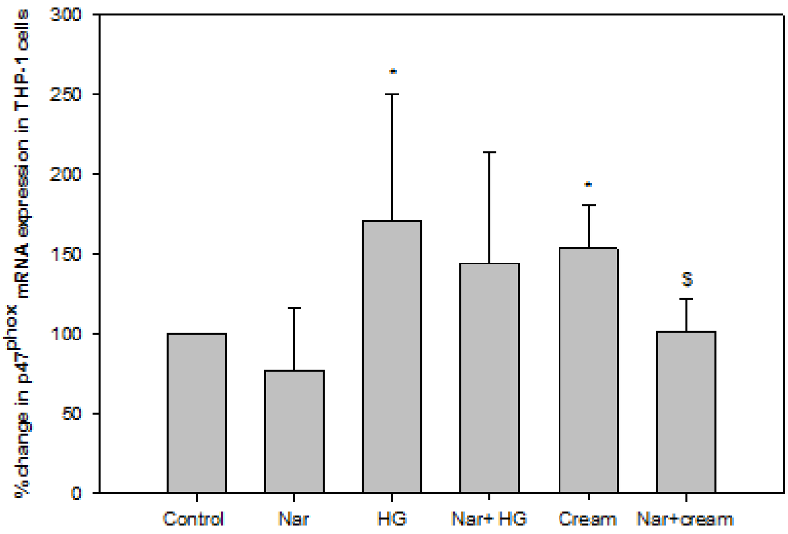


Figure 13: Percent change in mRNA expression ofp47^{phox} in THP-1 measured by RT-PCR (n=6); Data are means ± SEM.*:P<0.05 compared to control; $:P<0.05 comparing cream with Nar+cream, all the comparisons were made using paired t-test. Nar:Naringenin, HG: High concentration of glucose(15mM).

Table 6: Percent change in mRNA expression ofp47^{phox} in THP-1 measured by RT-PCR (n=6).

Sample	**% change**	***P*-value**
Naringein	↓ 23%	.178
15mM Glucose	↑ 71%	.038
Naringenin+15mM Glucose	↑ 45%	.113
Cream	↑ 54%	.005
Naringenin+cream	↑ 2%	.801

3. CCR-2 mRNA expression in THP-1 cells

Naringenin , naringenin with high concentration of glucose (15mM) and naringenin with cream resulted in a significant fall by 32±10% (p=0.015), 26±6% (P=0.004) and 34±7% (P=.015), respectively compared to control in CCR-2 mRNA expression (Table 7). However, challenging THP-1 cells with glucose (15mM) showed a trend of increase in the mRNA expression of CCR-2 gene (P=0.098), whereas cream caused no change. Naringenin with high concentration of glucose (15mM) caused a significant reduction by 42±9% (P=0.008) when compared to the expression of CCR-2 at 15mM of glucose alone (Figure 14).

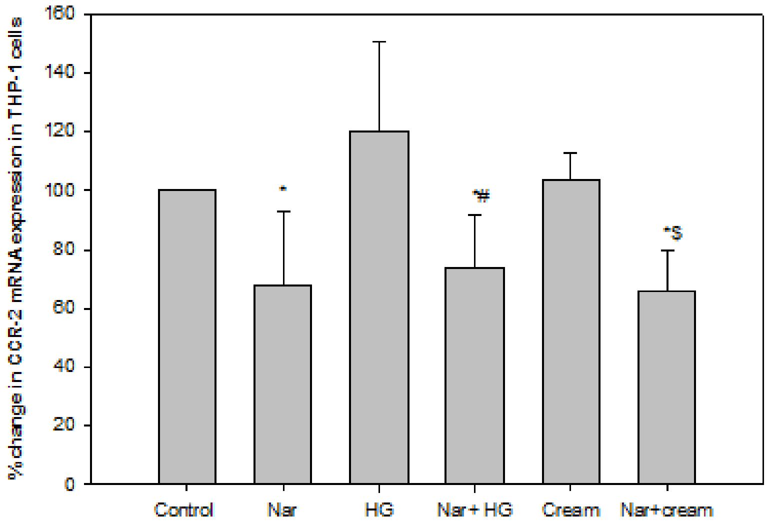


Figure 14: Percent change in mRNA expression of CCR-2 in THP-1 measured by RT-PCR (n=6); Data are means ± SEM. *:P<0.05 compared to control; #:P<0.05 comparing HG with Nar+HG; $:P<0.05 comparing cream with Nar+cream, all the comparisons were made using paired t-test. Nar:Naringenin, HG: High concentration of glucose(15mM).

Table 7: Percent change in mRNA expression of CCR-2 in THP-1 measured by RT-PCR (n=6)

Sample	**% change**	***P*-value**
Naringein	↓ 32%	.015
15mM Glucose	↑ 20%	.098
Naringenin+15mM Glucose	↓ 26%	.004
Cream	↑ 4%	.481
Naringenin+cream	↓ 34%	.015

4. MCP-1 mRNA expression in THP-1 cells

There was a significant reduction by 26±7% (P=0.01) in MCP-1 expression compared to control when incubating THP-1 cells with naringenin at low glucose conditions. Also, incubating naringenin with 15mM of glucose decreased the expression by 40±14% (p<0.001) compare to glucose alone (Table 8). There was no significant change in MCP-1 expression at 15mM of glucose. On the other hand, Cream significantly increased the expression of MCP-1 by 132±28% (P=0.031) (Figure 15).

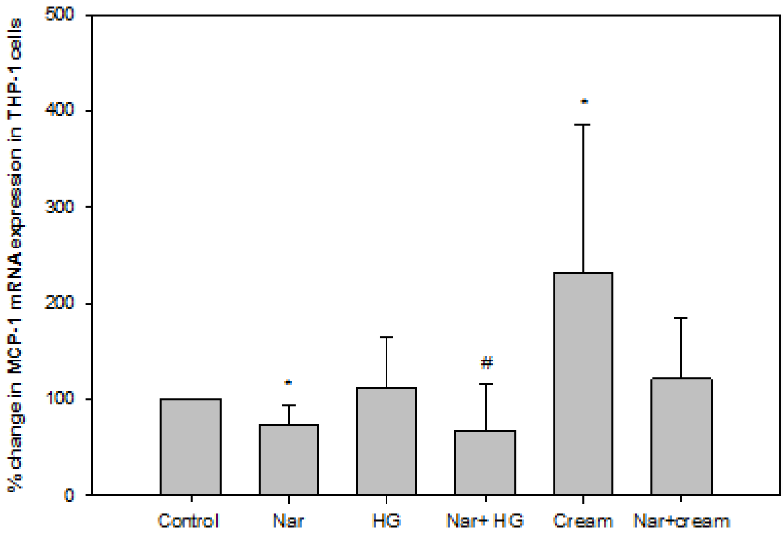


Figure 15: Percent change in mRNA expression of MCP-1 in THP-1 measured by RT-PCR (n=6); Data are means ± SEM. *:P<0.05 compared to control; #:P<0.05 comparing HG with Nar+HG, all the comparisons were made using paired t-test. Nar:Naringenin, HG: High concentration of glucose(15mM).

Table 8: Percent change in mRNA expression of MCP-1 in THP-1 measured by RT-PCR (n=6).

Sample	% change	*P*-value
Naringein	↓ 26%	.011
15mM Glucose	↑ 15%	.844
Naringenin+15mM Glucose	↓ 46%	.148
Cream	↑ 132%	.031
Naringenin+cream	↑ 22%	.445

5. HO-1 mRNA expression in THP-1 cells

HO-1 expression increased significantly with naringenin and naringenin with high concentration of glucose (15mM) by 41±13% and 92±40%, respectively (P<0.05) compared to control (Table 9). On the other hand, challenging THP-1 cells with 15mM of glucose or cream showed no statistically significant change. As for incubating naringenin with cream, HO-1 expression increased significantly by 128± 36% (P=0.016) (Figure 16).

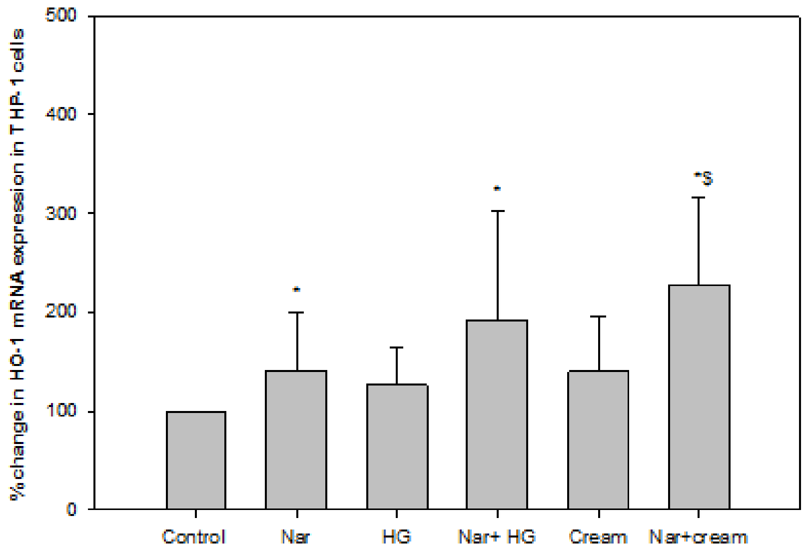


Figure 16: Percent change in mRNA expression of HO-1 in THP-1 measured by RT-PCR (n=6); Data are means ± SEM. *:P<0.05 compared to control; $:P<0.05 comparing cream with Nar+cream, all the comparisons were made using paired t-test. Nar:Naringenin, HG: High concentration of glucose(15mM).

Table 9: Percent change in mRNA expression of HO-1 in THP-1 measured by RT-PCR (n=6).

Sample	% change	*P*-value
Naringein	↑ 41%	.031
15mM Glucose	↑ 26%	.130
Naringenin+15mM Glucose	↑ 92%	.016
Cream	↑ 40%	.140
Naringenin+cream	↑ 128%	.016

6. TLR-4 mRNA expression in HAEC

Incubating HAEC with naringenin at basal condition (5mM Glucose) caused a significant reduction in TLR-4 expression by 52±9% (P=0.002) (Table 10). When these cells were incubated with high concentration of glucose (15mM), there was a significant increase by 48±8% (P=0.002) in TLR-4 expression whereas, cream incubation did not alter its expression (Figure 10). Also, challenging HAEC with 15mM of glucose or cream in the presence of naringenin caused a significant fall in TLR-4 expression by 38±8% (P=0.005) and 58± 7% (P= <0.001), respectively compared to Control (figure 17).

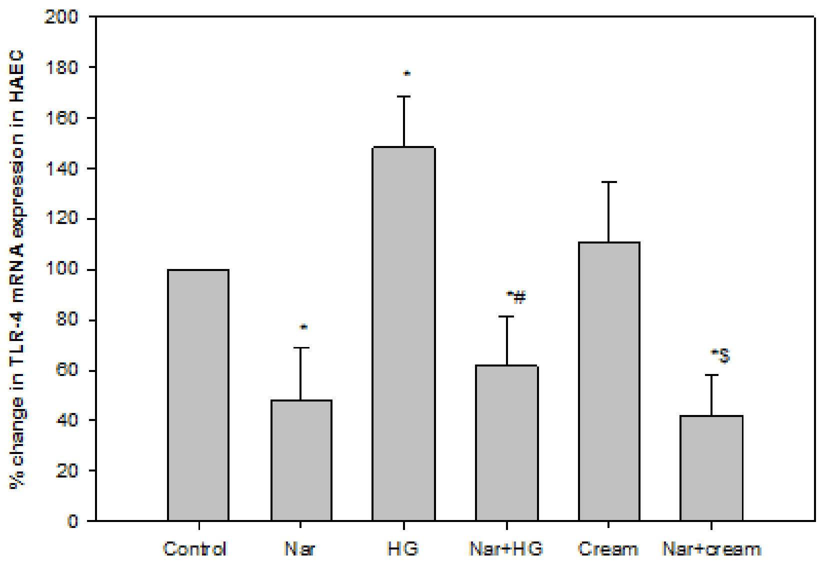


Figure 17: Percent change in mRNA expression of TLR-4 in HAEC measured by RT-PCR (n=6); Data are means ± SEM. *:P<0.05 compared to control; #:P<0.05 comparing HG with Nar+HG; $:P<0.05 comparing cream with Nar+cream, all the comparisons were made using paired t-test. Nar:Naringenin, HG: High concentration of glucose(15mM).

Table 10: Percent change in mRNA expression of TLR-4 in HAEC measured by RT-PCR (n=6).

Sample	**% change**	***P*-value**
Naringein	↓ 52%	.002
15mM Glucose	↑ 48%	.002
Naringenin+15mM Glucose	↓ 38%	.005
Cream	↑ 11%	.325
Naringenin+cream	↓ 58%	.001

7. HO-1 mRNA expression in HAEC

HO-1 expression increased significantly with naringenin alone by 101±30% (P=0.031) as well as when challenging HAEC with high concentration of glucose (15mM) or cream in the presence of naringenin by 35±5% and 220±28%, respectively (P = <0.001) (Table 11). However, incubating the cells with just high concentration of glucose (15mM) did not alter HO-1 expression. The percent change in HO-1 expression is much bigger with cream challenge in the presence of naringenin than the cream alone by 131±20% (P=<0.001) (Figure 18).

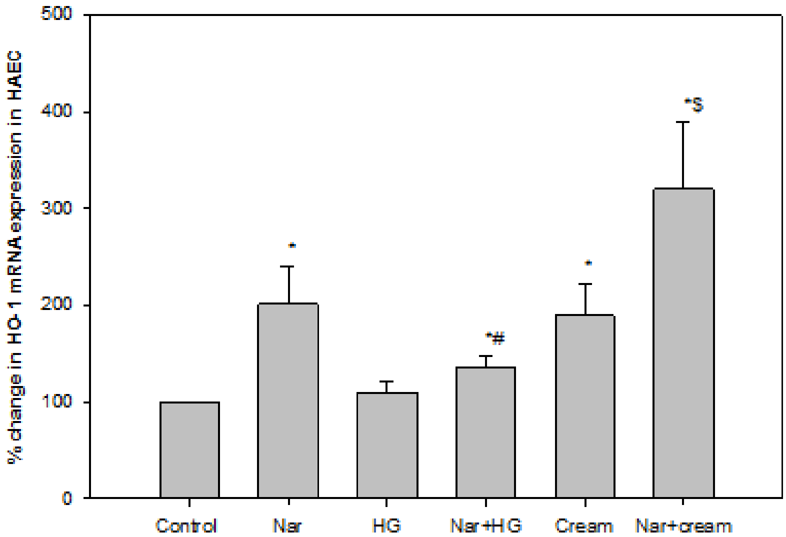


Figure 18: Percent change in mRNA expression of HO-1 in HAEC measured by RT-PCR (n=6); Data are means ± SEM. *:P<0.05 compared to control; #:P<0.05 comparing HG with Nar+HG; $:P<0.05 comparing cream with Nar+cream, all the comparisons were made using paired t-test. Nar:Naringenin, HG: High concentration of glucose(15mM).

Table 11: Percent change in mRNA expression of HO-1 in HAEC measured by RT-PCR (n=6).

Sample	% change	*P*-value
Naringein	↑ 101%	.031
15mM Glucose	↑ 9%	.133
Naringenin+15mM Glucose	↑ 35%	.001
Cream	↑ 74%	.031
Naringenin+cream	↑ 220%	.001

8. MCP-1 mRNA expression in HAEC

There was a significant reduction by 58±7% and 51±5% (P = <0.001) in MCP-1 expression when incubating HAEC with naringenin at low or high glucose concentrations, respectively (Table 12). On the other hand, challenging the cells with just high concentration of glucose caused no change in MCP-1 expression. There was a significant increase in MCP-1 expression with cream alone by 169±29% (P=0.002) which was suppressed by 94± 23% in the presence of naringenin (P=0.009) (Figure 19).

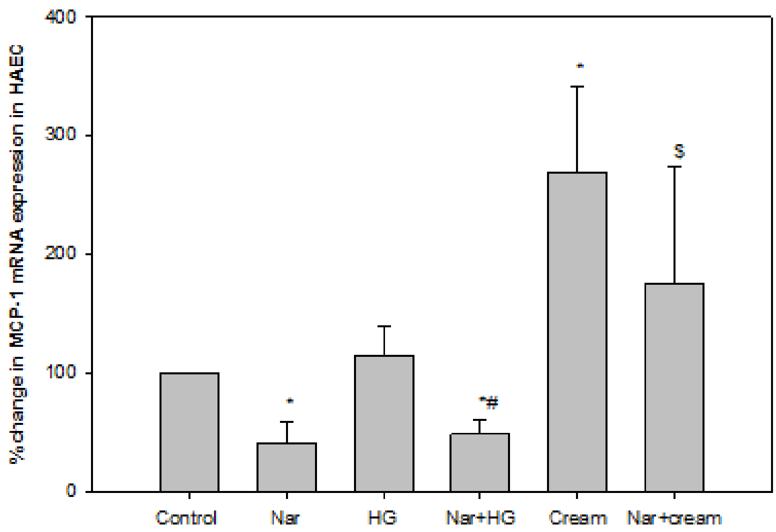


Figure 19: Percent change in mRNA expression of MCP-1 in HAEC measured by RT-PCR (n=6); Data are means ± SEM. *:P<0.05 compared to control; #:P<0.05 comparing HG with Nar+HG; $:P<0.05 comparing cream with Nar+cream, all the comparisons were made using paired t-test. Nar:Naringenin, HG: High concentration of glucose(15mM).

Table 12: Percent change in mRNA expression of MCP-1 in HAEC measured by RT-PCR (n=6)

Sample	**% change**	***P*-value**
Naringein	↓ 58%	.001
15mM Glucose	↑ 11%	.156
Naringenin+15mM Glucose	↓ 51%	.001
Cream	↑ 169%	.002
Naringenin+cream	↑ 75%	.121

9. VCAM-1 mRNA expression in HAEC

Challenging HAEC with cream or high concentration of glucose (15mM) resulted in significant increases in VCAM-1 expression by 78±18% (P=0.007) and 46±14% (P=0.023), respectively (Table 13). As for the effect of naringenin, there was only a trend towards reducing the expression of VCAM-1 when naringenin was added at low or high glucose concentrations or in the presence of cream (Figure 20).

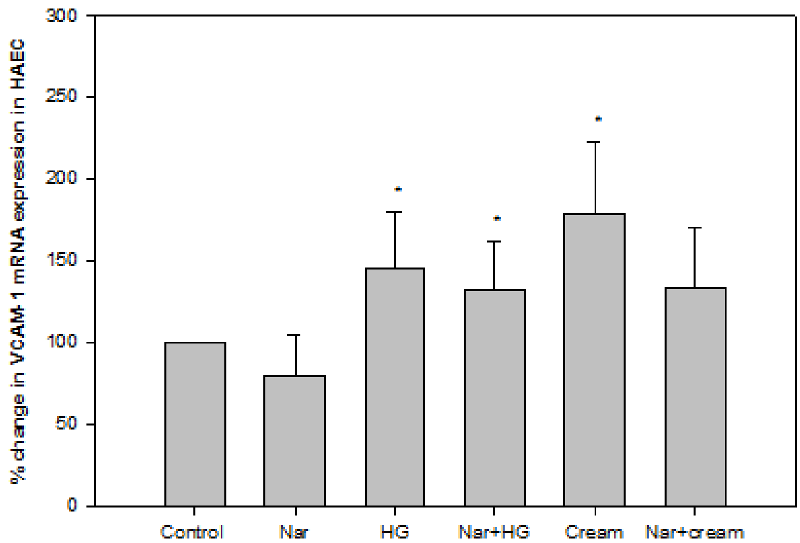


Figure 20: Percent change in mRNA expression of VCAM-1 in HAEC measured by RT-PCR (n=6); Data are means ± SEM. *:P<0.05 compared to control; #:P<0.05 comparing HG with Nar+HG; $:P<0.05 comparing cream with Nar+cream, all the comparisons were made using paired t-test. Nar:Naringenin, HG: High concentration of glucose(15mM).

Table 13: Percent change in mRNA expression of VCAM-1 in HAEC measured by RT-PCR (n=6).

Sample	**% change**	***P*-value**
Naringein	↓ 20%	.112
15mM Glucose	↑ 46%	.023
Naringenin+15mM Glucose	↑ 32%	.050
Cream	↑ 78%	.007
Naringenin+cream	↑ 33%	.085

10. NADPH oxidase $P47^{phox}$ protein content

We measured the protein content of the NADPH oxidase subunit, $p47^{phox}$, as an oxidative stress marker in THP-1 cell. There was no significant change in $p47^{phox}$ protein quantity when incubating the THP-1 cells with naringenin or challenging the cells with either high concentration of glucose or cream in the presence of naringenin (Figure 21).

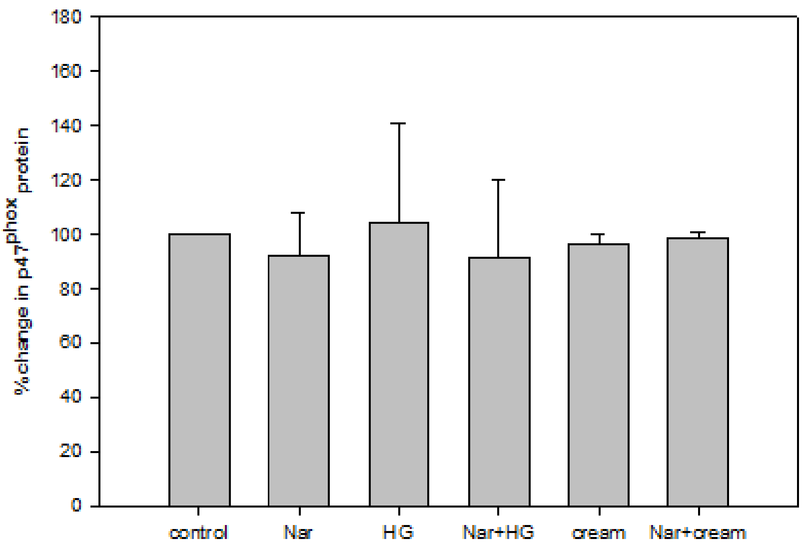


Figure 21: Percent change in protein quantity of the NDPH oxidase subunit, p47^{phox}, by western blotting (n=6). Data are means ± SEM.All the comparisons were made using paired t-test. Nar:Naringenin, HG: High concentration of glucose(15mM).

DISCUSSION

Our data clearly show that naringenin suppresses glucose and cream induced IL-1β and CCR-2 expression in THP-1 cells. In addition, the TLR-4 was also suppressed in HAEC cells. On the other hand, naringenin increased HO-1 expression (antioxidant enzyme) in THP-1 and HAEC cells. Thus, this citrus flavonoid exerts antioxidant and anti-inflammatory effects. Therefore, these actions may have a potential antiatherogenic effect. Further studies are required on the effect of naringenin on atherosclerosis *in vivo* and at different doses.

Atherosclerosis is considered to be a chronic inflammatory disease and many cytokines, chemokines and adhesion molecules participate in the development of atherosclerosis. IL-1β, a proinflammatory cytokine produced by activated macrophages, has been implicated in the pathogenesis of atherosclerosis in previous studies (Kleemann *et al.,* 2008). Our data show that naringenin significantly suppressed glucose and cream induced IL-1β expression in THP-1 cells by 60±17% and 19±23%, respectively. The ability of naringenin to suppress IL-1β was also seen when inducing macrophages and whole blood with lipopolysaccharide (Bodet *et al*., 2008). This is consistent with data from Taiwanese study (Tsai *et al*., 2012) which reported that supplementing the diet of diabetic lab mice with 2% naringenin reduced levels of various inflammatory markers, including IL-1β by 45%. As expected, both cream and high concentration of glucose resulted in a significant increase IL-1β expression in THP-1 cells which is consistent with Deopurkar *et al*., (2010) observation on the effect of glucose and cream intake on IL-1β expression in monocytes in normal subjects. These observations are clinically important because

macronutrients induced inflammation has been shown to play an important role in the pathogenesis of diabetes and atherosclerosis. On the basis of the above, naringenin can be considered as a potent inhibitor of IL-1β cytokine and may have a potential anti-inflammatory effect in atherosclerosis. Further investigation is needed to know the effect of naringenin on other proinflammatory cytokines as well as on the intranuclear binding of NFkB, a major proinflammatory transcription factor that regulates the transcription of many proinflammatory cytokines including IL-1β.

As for $p47^{phox}$ mRNA expression, the cardinal subunit of nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), the enzyme that converts molecular O_2 into O_2^- radical, our results show that cream and 15mM of glucose caused a significant increase in $p47^{phox}$ expression in THP-1 cells by 54±11% and 71±28%, respectively. This result support the observation that high-fat, high-carbohydrate meal increase ROS generation in monocyte (Aljada *et al*., 2004). Since the increase in $p47^{phox}$ expression will probably cause an increase in NADPH oxidase activity which can lead to the generation of more ROS. The fact that macronutrients induce oxidative stress is of clinical importance because oxidative stress is hypothesized to be an important contributor to the pathophysiology of vascular diseases such as atherosclerosis, hypertension, and diabetic microvascular disease. Several studies demonstrated the necessity of NADPH oxidase for normal atherogenic lesion progression in $ApoE^{(-/-)}$ mice (Barry-Lane *et al*., 2001). On the other hand, naringenin in the presence of cream resulted in suppression of $p47^{phox}$ expression whereas naringenin in the presence of high concentration of glucose caused no statistically significant change in $p47^{phox}$expression.

These results demonstrate that the anti-oxidant activities of naringenin are in part mediated by $p47^{phox}$suppression.

The chemokine receptor, CCR2, is the receptor for MCP-1 and is primarily expressed on hemopoietic cells. It plays an important role in the recruitment of monocytes/macrophages and T cells. Recruitment of leukocytes, especially monocytes, is central to the pathology of atherosclerosis. It has been shown that CCR2 $^{(-/-)}$mice have defects in leukocyte adhesion and monocyte/macrophage recruitment (Kuziel *et al.*, 1997).Also, the absence of CCR2 on macrophages protected those mice from developing atherosclerosis. Therefore, we were interested in studying the effect of naringenin on this chemokine receptor in cells that a monocytic cell line (THP-1 cells). Our results show that incubating THP-1 cells with naringenin suppressed CCR-2 expression both a basal levels and when cream or 15mM of glucose was added to the cells. Although this is a good indicator that naringenin can be used as a suppressor for CCR-2 mRNA expression we still need to know its effect on CCR-2 protein expression. On the other hand, we were surprised that neither cream nor high concentration of glucose caused an increase in the expression of CCR-2 although it has been shown that macronutrients induce inflammation. It is possible that longer incubation with cream and glucose are needed or that macronutrients exert their atherogenic effect via pathways that does not modulate CCR-2 expression. Clearly more experiments are needed on the effect of naringenin on CCR-2 in animal models as well as in human.

We were interested too in the effect of naringenin on the chemokine monocyte chemoattractant protein-1 (MCP-1), the ligand for CCR-2 receptor. MCP-1 is an inflammatory chemotactic mediator that plays a role in leucocyte recruitment into the

subendothelial space. It has been shown that MCP-1 is highly expressed in human atherosclerotic lesions and that the over expression of this chemokine results in an increase in macrophages number within the lesions (Nelken *et al*., 1991). On the other hand, mice lacking the ligand MCP-1 showed a decrease in atherosclerotic lesion size and a significant reduction in macrophages in lesions (Gu *et al*., 1998). Our data show that incubating THP-1 cells with nararingin at basal condition suppresses MCP-1 expression. Naringenin also suppressed MCP-1 expression in presence of 15mM of glucose which did not induce MCP-1 expression. The ability of naringenin to decrease the expression of MCP-1 is consistent with reports that naringenin decreased the expression of MCP-1 in rabbits fed high cholesterol diet supplemented with 0.05% naringenin for 8 weeks (Lee *et al*., 2001).The incubation of THP-1 cells with high concentration of glucose for one hour caused no change in MCP-1 expression. This observation didn't surprise us because Takaishi *et al.,* (2003) showed that chronic incubation of human umbilical vein endothelial cells with high glucose increased mRNA expression and production rate of MCP-1 in a time (1–7 days)- and concentration (10–35 mM)-dependent manner. Therefore, it is possible that increasing the incubation time will result in an increase in MCP-1 expression in our cells. As for the effect of cream, there was a significant increase in the expression of MCP-1. This observation is attributed to what has been previously shown that cream intake induces inflammation (Deopurkar *et al*., 2010) and indicates the potential atherogenic effect of cream.

We also measured the mRNA expression of MCP-1 in HAEC cells. These cells showed a significant increase in MCP-1 expression only when challenged with cream but not with glucose. The addition of naringenin resulted in a significant decrease in MCP-1

expression at both low and high glucose concentrations and in the presence of cream. This indicated that naringin effect on HAEC is independed of the cells activation level and is not controlled by inducible mechanisms. Our results show that both cell types (THP-1 and HAEC) have the same MCP-1 mRNA expression pattern when performing our experiments with naringenin. Considering the suppressive effect of naringenin on the expression of both CCR-2 and its ligand MCP-1 it is possible that naringenin has antiatherogenic activity through suppressing monocytes recruitment.

Since HO-1 has anti-inflammatory and anti-oxidant effects, we investigated the effect of naringenin on HO-1 gene expression in macronutrient induced oxidative stress and inflammation conditions in both cell types (THP-1 and HAEC). HO-1 is the inducible isoform of the first and rate-limiting antioxidant enzyme of heme degradation, which produces biliverdin and the signaling gas carbon monoxide (CO) (Immenschuh S and Schröder H., 2006). Several studies investigated the link of HO-1 to atherosclerosis because it is a condition associated with oxidative stress, a potent inducer of HO-1 gene expression. It has been shown that induction of HO-1 inhibits the monocytes transmigration induced by mildly oxidized LDL in a co-cultured human endothelial and smooth muscle cells (Ishikawa *et al*., 2001). Also, *in vivo* studies in LDL-receptor knock-out mice and Watanabe heritable hyperlipidemic rabbits showed that HO-1 inhibits atherosclerotic lesion formation (Ishikawa *et al*., 2001). Our results show that HO-1 mRNA expression in THP-1 as well as in HAEC cells was significantly induced by naringenin both at basal conditions and when challenged with 15mM of glucose or cream. Also, our results show clearly that challenging the cells with cream in the presence of naringenin results in a higher HO-1 expression levels when comparing it with

the effect of 15mM of glucose in the presence of naringenin. It is possible that cream results in more oxidative stress than 15mM of glucose as has been previously demonstrated (Deopurkar *et al*., 2010).

It is interesting that there was no significant change in the expression of HO-1 when challenging THP-1 cells with either glucose or cream alone. Two recent studies showed opposite effects of glucose on HO-1 expression, the first one demonstrated that microvessel endothelial cells exposed to 33mM glucose decrease HO activity and HO-1 protein (Abraham *et al*., 2003) while Iori *et al.,*(2008) showed that human umbilical vein endothelial cells challenged with 10 mM glucose increase HO-1 protein expression and HO activity whereas at 20 mM, there was no change in protein content and activity relative to at 5.5 mM glucose. On the other hand, in our study cream but not glucose resulted in a significant increase in HO-1 expression in HAEC. These results can be explained by the fact that different types of cells may have different response to the same stimulus. It is important to know the effect of naringenin on mRNA and protein expression of other antioxidant enzymes as well as on the binding activity of Nrf-2, a transcription factor that regulates the transcription of many antioxidant enzymes.

We also studied the effects of naringenin on the expression of another important receptor, TLR-4. TLR-4 is a specific receptor for lipopolysaccharide (LPS) or endotoxin from gram negative bacteria and plays an important role in endotoxemia. TLR-4 is also involved in the pathogenesis of atherosclerosis, diet induced obesity and the related insulin resistance. It has been shown that TLR-4 is expressed in atherosclerotic plaques and that TLR-4 deletion protects mice from atherosclerosis and insulin resistance induced by a high fat diet (Shi *et al*., 2006, Li *et al*., 2007, Kim *et al*., 2007). Our results show that

naringenin caused a significant decrease in TLR-4 expression in HAEC cells both in basal and challenged conditions. Ghanim *et al*., (2010) showed that the intake of orange juice, which contains two major flavonoids hesperitin and naringenin, with-high fat, high-carbohydrate meal suppresses TLR-4 mRNA expression and protein in monocyte, *in vivo*. On the other hand we were surprised that 15mM of glucose but not cream resulted in a significant increase in TLR-4expression. This observation is in contrast to what has been previously demonstrated by Deopukar *et al*, (2010) in mononuclear cells (MNC) showing that cream but not glucose causes an increase in TLR-4 expression in MNC. It has been shown previously that glucose induces TLRs expression in monocytes in type 2 diabetes patients (Dasu *et al*., 2010). Differences in testing conditions and cell type may explain such differences in outcomes. Therefore, further studies are needed on the effect of naringenin on TLR-4 expression, *in vivo*.

As we know inflammation participates in all stages of atherosclerosis starting with the attachment of monocyte to endothelial cells through the vascular cell adhesion molecule -1 (VCAM-1) expressed on endothelial cells to its ultimate endpoint, thrombotic complication. Endothelial cells normally resist leukocyte adhesion because they do not express VCAM-1 under baseline conditions , however, triggering these cells with high-saturated-fat diet, smoking, hypertension, hyperglycemia, obesity, or insulin resistance result in an initiation of VCAM-1 expression in these cells. Therefore, we studied the effect of naringenin on the expression of VCAM-1 in HAEC cells using high concentration of glucose and cream as inducers for VCAM-1 expression. Our results show that naringenin did not inhibit the increase in VCAM-1 expression by high glucose as well as cream. In contrast to our results Lee *et al* (2001) showed that 0.05% of

naringenin caused downregulation in VCAM-1 expression in thoracic aorta of high cholesterol fed rabbits.

In conclusion, naringenin suppresses high glucose and cream induced expression of IL-1β as well as CCR-2 and its ligand (MCP-1) in THP-1 cells. Also, TLR-4 and MCP-1 expressions are suppressed in HAEC. However, HO-1 expression, which is an antioxidant enzyme, increased significantly in both THP-1 and HAEC cells. These neutralizing effects of naringenin on macronutrient induced oxidative stress and inflammation may have a potential antiatherogenic effects.

CONCLUSIONS:

On the basis of the results of this study, the following conclusions can be made:

1. Naringenin suppresses macronutrient induced expression of IL-1β, aproinflammatory cytokine, in THP-1 cell. This effect is consistent with anti-inflammatory concept of naringenin.

2. The inhibitory effect of naringenin on CCR-2 and MCP-1 mRNA expression leads to the speculation that the anti-atherogenic effects of naringenin may be mediated by the prevention of macrophages recruitment to the subendothelial space.

3. Naringenin reduces TLR-4 mRNA expression in HAEC. This effect is relevant to the pathogenesis of atherosclerosis since TLR-4 expression is observed in atherosclerotic plaques.

4. HO-1 mRNA expression increases with naringenin in both THP-1 and HAEC cell lines. This gene is an antioxidant enzyme and therefore, naringenin has antioxidant property.

5. Naringenin did not have any effect on $p47^{phox}$ protein quantity. However, longer incubation periods or higher naringenin concentrations may be necessary for naringenin effect to be detectable.

6. Naringenin reduces the expression of VCAM-1 adhesion molecule when cells are challenged with glucose but not cream.

RECOMANDATIONS

On the basis of the conclusions made, the following recommendations can be made:

1. Increase naringenin concentrations and incubation periods.

2. Measure the DNA binding activity of the proinflammatory transcription factor NFκB as well as Nrf2, a transcription factor that regulates the transcription of many antioxidant enzymes.

3. Determine the mRNA expression level for other cytokines, chemokines and adhesion molecules.

4. Co-culture THP cells and endothelial cells under basal and macronutrient rich conditions in the presence of naringenin.

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تأثير مركبات الفلافونويد (النرنجينن) على الأكسدة و الالتهابات: دوره في تصلب الشرايين

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ملخص

ارتبطت الاكسدة والالتهابات مع العديد من الأمراض مثل السكري من النوع الثاني و مقاومة الانسولين وتصلب الشرايين. فقد تبين أن المواد الغذائية الرئيسية تحث الاكسدة و الالتهابات. الهدف من هذه الدراسة هو تحديد تأثيرالنرنجينن، وهي حمضيات الفلافونويد الموجودة في الجريب فروت والبرتقال، على الاكسدة والالتهابات التي تسببها المواد الغذائية الرئيسية (الجلوكوز أو الدهون) في الخلايا المشاركة في تصلب الشرايين مثل الوحيدات والخلايا المبطنة لجدار الأورطة.

استخدم في هذه الدراسة نوعين من الخلايا المشارك في تصلب الشرايين حيث تم اضافة 15 ملي مول من الجلوكوز أو 20 ميكرو ليتر /مل من الكريمة لكل من الخلايا المبطنة لجدار الأورطة (HAEC) و الوحيدات (THP-1) و ذلك في حالة وجود أو عدم وجود 100ميكرو مول من النرنجينن. و من ثم تم قياس تعبير الحمض النووي الريبي المرسال للعديد من الجينات مثل IL-1β، $p47^{phox}$، HO-1، MCP-1، CCR-2، TLR-4 وVCAM-1 بواسطة تفاعل البلمرة المتسلسل ذات الزمن الحقيقي لكل من الوحيدات و الخلايا المبطنة لجدار الأورطة. كذلك تم قياس تعبير بروتين $p47^{phox}$ في الوحيدات باستخدام لطخة ويسترن (western blot) .

استطاع مركب النرنجين تثبيط تأثير الأغذية الرئيسية (الجلوكوز و الكريمة) المحفزة لتعبير كلا من الجينات التالية: IL-1 β، CCR-2، وMCP-1 في الوحيدات. وبالإضافة إلى ذلك ، فان مستقبل TLR-4 تم تثبيطه أيضا في الخلايا المبطنة لجدار الأورطة. و من ناحية أخرى، قام مركب النرنجين بزيادة تعبير HO-1 (أنزيم مضاد للأكسدة) بشكل كبير في الوحيدات والخلايا المبطنة لجدار الأورطة. . لم يكن هناك تغيير في التعبير عن بروتين $p47^{phox}$ في الوحيدات. ومع ذلك، خفض مركب النرنجين التعبير عن VCAM-1 في الخلايا المبطنة لجدار الأورطة في حالة وجود الجلوكوز ولكن ليس الكريمة.

بناءا على نتائج هذه الدراسة فانه يمكن الستخلاص بأن مركب النرنجين لديه القدرة على معادلة تأثير الأغذية الرئيسية المسببة للأكسدة و الألتهابات. لذلك من المحتمل أن يكون لهذا المركب تأثير مضاد لتصلب الشرايين.